BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

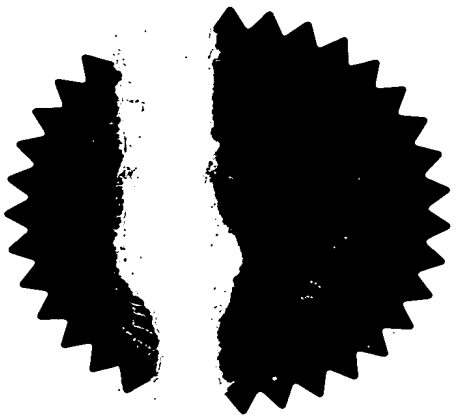
I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that by virtue of an assignment registered under the Patents Act 1977, the application is now proceeding in the name as substituted.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed

Dated 16 August 2000

GB9806113.8

By virtue of a direction given under Section 30 of the Patents Act 1977, the application is proceeding in the name of

BIOGEMMA UK LIMITED,
200 Cambridge Science Park,
Milton Road,
CAMBRIDGE,
CB4 OGZ,
United Kingdom

[ADP No. 07622137001]

Patents Form 1/77

1/77

Patents Act 1977
(Rule 16)

The Patent Office

24MAR98 E347683-1 D00056
P01/7700 25.00 - 9806113.8

THE PATENT OFFICE
A
20 MAR 1998
LONDON

The Patent Office

Newport
Gwent NP9 1RH

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

Cardiff Road

1. Your reference

KVC/P20895GB

2. Patent application number
(The Patent Office will fill in this part)

9806113.8

3. Full name, address and postcode of the or of each patent applicant *(underline all surnames)*

NICKERSON BIOCEM LIMITED
Cambridge Science Park
Milton Road
Cambridge, CB4 4GZ
UK

SECTION 30 (1977 ACT) APPLICATION FILED 17/2/98.

Patents ADP number *(if you know it)* 07408842001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

CONTROL

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

KILBURN & STRODE
20 RED LION STREET
LONDON
WC1R 2PJ

Patents ADP number *(if you know it)* 125001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application number	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

YES

a) any applicant named in part 3 is not an inventor, or

b) there is an inventor who is not named as an applicant, or

c) any named applicant is a corporate body. See note (d))

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the sam document

Continuation sheets of this form	:	
Description	:	22
Claim(s)	:	4
Abstract	:	-
Drawing(s)	:	4 +4

10. If you are also filing any of the following, state how many against each item.

Priority documents	:
Translations of priority documents	:
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	:
Request for preliminary examination and search *(Patents Form 9/77)*	:
Request for substantive examination *(Patents form 10/77)*	:
Any other documents *(please specify)*	:

11. I/We request the grant of a patent on the basis of this application.

Signature: Kilburn & Strode — Date: 20 March 1998

12. Name and daytime telephone number of person to contact in the United Kingdom

Kristina Cornish
Tel: 0171-539 4200

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink oryou may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered `Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

CONTROL

This invention relates to a novel plant nucleic acid sequence and protein. The sequence and protein are useful in the control of plant dehiscence and in the production of male sterile plants.

The production of seed is an important developmental process in all higher plants. In oilseed rape (*Brassica napus*) following abscission of floral parts, pods or siliques are formed which contain 15-30 seeds. Around 50-70 days after anthesis (DAA) the pods become susceptible to shatter, a process that serves to expel the mature seeds into the surrounding environment. In the days leading to dehiscence, an array of anatomical, molecular and biochemical changes take place, thus preparing both seed and pod for the event. Shatter eventually occurs as a result of a combination of factors including: the creation of tensions within the pod between the lignified valve edge cells of the endocarp and the unlignified dehiscence zone (DZ) cells, weakening of the DZ cell walls by hydrolytic enzyme activity and ultimately due to physical forces such as strong winds or harvesting machinery.

Pod development in *B. napus* can be segmented into three stages. In the first stage, which occurs 0-20 DAA, the newly formed siliques, consisting of two seed-containing carpels separated by a false septum and a replar region, grow to their full length of around 10cm. The seeds begin to grow when the pods are virtually full size [Hocking and Mason, 1993]. Between 10 and 20 DAA the cells in the replar region begin to differentiate into replar cells, large valve edge cells and form a distinct region, 1-3 cells wide, comprising the DZ [Meakin and Roberts, 1990a].

The second stage occurs between 20 and 50 DAA. From 20 DAA, in conjunction with termination of pod elongation, secondary cell wall material is deposited in the

valve edge cells, and the replar cells become increasingly lignified. The DZ cells do not exhibit thickening of the cell wall. A progressive shrinkage and loss of organelles is apparent in the DZ cells from 40 DAA onwards and eventually these cells separate completely due to hydrolysis of the middle lamella [Meakin and Roberts, 1990a]. In the final stage of pod development, which occurs 50-70 DAA, the lignified cells undergo senescence and the necessary tensions are created so that the desiccated pod, containing mature seed, eventually shatters.

Molecular studies of the penultimate stage of pod development have revealed a spatial and temporal correlation between the up-regulation of a number of mRNAs and pod dehiscence in *B. napus*. These mRNAs encode a polygalacturonase (PG) and a proline-rich protein (SAC51). Further analysis of the expression of the PG following fusion of a pod-specific *Arabidopsis thaliana* PG promoter to GUS, Jenkins et al., 5th International Conference of Plant Molecular Biology (1997), Abstract 310), has revealed that reporter gene expression is restricted precisely to the layer of cells comprising the pod DZ in transgenic *B. napus*. From 40 DAA, Meakin and Roberts (1990b) reported a progressive increase in β-1,4-glucanase (cellulase) activity in the DZ.

It is understood that the processes of dehiscence and abscission are not regulated by the same environmental or chemical signals, but that they involve controlled degradation of cell wall material and cell separation in a distinct group of cells. Both ethylene and indole-3-acetic acid (IAA) appear to be important regulators of the timing of the abscission process but the role of these plant hormones in dehiscence is less clearly defined. The increase in cellulase activity has been shown to correlate with a rise in the production of ethylene, mainly from the seed, which peaks at around 40 DAA [Meakin and Roberts, 1990b; Johnson-Flanagan and Spencer, 1994].

Developmental processes, such as pod dehiscence, which involve highly regulated and controlled expression of an array of different genes at a precise time and cellular location, clearly require an intricate signal transduction network.

Further and improved genetic elements to control plant processes in this area are constantly desired. We describe the isolation, for the first time, of a plant cDNA (CW1) encoding an individual response regulator protein, the expression of which is closely correlated with dehiscence of fruit in *B. napus*. CW1 has a role in the ability to control molecule signaling during the events leading to shatter and thus to control pod shatter in plants.

According to a first aspect of the invention there is provided nucleic acid encoding a signal transduction protein involved in the process of dehiscence. Such a signal transduction protein has never previously been described in plant dehiscence.

In this text, the term "involved in the process of dehiscence" means any nucleic acid encoding any protein which has an effect in the dehiscence process, in particular a protein involved in an MAP Kinase cascade or any other protein which results in changes in the expression of genes involved in dehiscence, such as upregulation of genes encoding polygalacturonase, cellulase, senescence-related proteins and/or downregulation of genes encoding for proteins involved in cells wall biosynthesis. Preferably the protein is involved in the process of dehiscence which involves the production of a hydrolytic enzyme such as polygalacturonase or cellulase.

The nucleic acid of the first aspect of the invention may be a nucleic acid which is naturally expressed in a dehiscence zone. Such a nucleic acid will most accurately reflect nucleic acid naturally expressed in a plant. Preferably the dehiscence zone is a pod (also termed "siliques"), anther and/or funiculus dehiscence zone. Preferably

the plant is a member of the Brassica family, maize or wheat or soyabean.

A second aspect of the invention provides nucleic acid encoding a protein wherein the protein:

a) comprises the amino acid sequence shown in figure 1 or;

b) has one or more amino acid deletions, insertions or substitutions relative to a protein as defined in a) above, but has at least 40% amino acid sequence identified therewith; or

c) is a fragment of a protein as defined in a) or b) above, which is at least 10 (preferably 20 or 30) amino acids long.

The skilled person will appreciate that various changes can sometimes be made to the amino acid sequence of a protein (which has a desired property) to produce variants (often known as "muteins") which still have said property. Such variants of the protein describe in a, b and c above are within the scope of the present invention and are discussed in greater detail below in sections *(i)* to *(iii)*. They include allelic and non-allelic variants.

(i) Substitutions

An example of a variant of the present invention is a polypeptide as defined in a, b or c above, apart from the substitution of one or more amino acids with one or more other amino acids.

The skilled person is aware that various amino acids have similar characteristics. One or more such amino acids of a protein can often be substituted by one or more

other such amino acids without eliminating a desired property of that protein.

For example, the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids that can often be substituted for one another include phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulphur containing side chains).

Substitutions of this nature are often referred to as "conservative" or "semi-conservative" amino acid substitutions.

(ii) Deletions

Amino acid deletions can be advantageous since the overall length and the molecular weight of a polypeptide can be reduced whilst still retaining a desired property. This can enable the amount of protein required for a particular purpose to be reduced. Proteins according to the present invention, which have such deletion(s) are useful. They may interfere with the normal functioning of CW1; that is, they may act as dominant negative mutations preventing normal CW1 functioning and thus be of particular value, for example, in reducing pod shatter.

The amino acid sequence shown in figure 1 has various functional regions. For particular applications of the present invention, one or more of these regions may not

be needed and may therefore be deleted.

(iii) Insertions

Amino acid insertions relative to a polypeptide as defined in a, b or c above can also be made. This may be done to alter the nature of the protein (e.g. to assist in identification, purification, or expression, as explained below in relation to fusion proteins).

Changes in the protein according to the present invention can produce versions of the protein that are constitutively active. If a protein of the present invention acts on an inhibitor of the release of hydrolytic enzymes, then a constitutively active version would prevent or reduce pod shatter

A protein according to any aspect of the invention may have additional N-terminal and/or C-terminal amino acid sequences. Such sequences can be provided for various reasons. Techniques for providing such sequences are well known in the art. They include using gene-cloning techniques to ligate together nucleic acid molecules encoding polypeptides or parts thereof, followed by expressing a polypeptide encoded by the nucleic acid molecule produced by ligation.

Additional sequences may be provided in order to alter the characteristics of a particular polypeptide. This can be useful in improving expression or regulation of expression in particular expression systems. For example, an additional sequence may provide some protection against proteolytic cleavage. This has been done for the hormone somatostatin by fusing it at its N-terminus to part of the β galactosidase enzyme (Itakwa *et al.*, *Science* **198**: 105-63 (1977)).

Additional sequences can also be useful in altering the properties of a polypeptide to

aid in identification or purification.

For example, a signal sequence may be present to direct the transport of the polypeptide to a particular location within a cell or to export the polypeptide from the cell. Hydrophobic sequences may be provided to anchor a polypeptide in a membrane. Thus the present invention includes within its scope both soluble and membrane-bound polypeptides.

Preferably, the nucleic acid according to the second aspect of the invention encodes a signal transduction protein or a functional portion thereof involved in the process of dehiscence. All preferred features of the first aspect of the invention also apply to the second.

The term protein used in this text means, in general terms, a plurality of amino acid residues joined together by peptide bonds. It is used interchangeably and means the same as polypeptide or peptide.

The nucleic acid according to the first or second aspect of the invention preferably comprises the sequence set out in figure 1, or a fragment thereof which is at least 30 bases long. While this nucleic acid is the preferred nucleic acid of the invention, it is well known to those persons skilled in the art that because of the nucleic acid "degenerate code" which encodes nucleic acids, a considerable number of variations in nucleic acid sequence can be used to encode for proteins according to the first or second aspects of the invention.

The nucleic acid of the first or second aspects of the invention may be isolated or recombinant and may be in substantially pure form. The nucleic acid may be antisense to nucleic acid according to the first or second aspects of the invention. As

understood by the person skilled in the art introducing the coding region of a gene in the reverse orientation to that found in nature (antisense) can result in the downregulation of the gene and hence the production of less or none of the gene product. The transcribed antisense DNA is capable of binding to and destroying the function of the sense RNA of the sequence normally found in the cell, thereby disrupting function. Antisense nucleic acid may be constitutively expressed, but is preferably limited to expression in those zones (dehiscence) in which the naturally occurring nucleic acid is expressed.

The nucleic acid according to the first or second aspects of the invention preferably include a promoter or other regulatory sequence which controls expression of the nucleic acid. Promoters and other regulatory sequences which control expression of a nucleic acid in dehiscence zones are known in the art, for example described in WO96/30529 and WO94/23043. Further promoters or other regulatory sequences can be identified and can also include the promoter or other regulatory sequence which controls expression of a nucleic acid as set out in figure 1. The person skilled in the art will know that it may not be necessary to utilize the whole promoter or other regulatory sequence. Only the minimum essential regulatory elements may be required and in fact such elements can be used to construct chimeric sequences or promoters. The essential requirement is, of course, to retain the tissue and/or temporal specificity.

The nucleic acid according to the first or second aspects of the invention may be in the form of a vector. The vector may be a plasmid, cosmid or phage. Vectors frequently include one or more expressed markers which enable selection of cells transfected (or transformed: the terms are used interchangeably in this text) with them and preferably, to enable a selection of cells containing vectors incorporating heterologous DNA. A suitable start and stop signal will generally be present and if

the vector is intended for expression, sufficient regulatory sequences to drive expression will be present. Nucleic acid according to the first and second aspects of the invention is preferably for expression in plant cells and thus microbial host expression is perhaps less important although not ruled out. Microbial host expression and vectors not including regulatory sequences are useful as cloning vectors.

A third aspect of the invention relates to a cell comprising nucleic acid according to the first or second aspects of the invention. The cell may be termed as "a host" which is useful for manipulation of the nucleic acid, including cloning. Alternatively, the cell may be a cell in which to obtain expression of the nucleic acid, most preferably a plant cell. The nucleic acid can be incorporated by standard techniques known in the art in to cells. Preferably nucleic acid is transformed in to plant cells using a disarmed Ti plasmid vector and carried by an Agrobacterium by procedures known in the art, for example as described in EP-A-0116718 and EP-A-0270822. Foreign nucleic acid can alternatively be introduced directly into plant cells using an electrical discharged apparatus or by any other method that provides for the stable incorporation of the nucleic acid into the cell. Preferably the stable incorporation of the nucleic acid is within the nucleic DNA of any cell preferably a plant cell. Nucleic acid according to the first and second aspects of the invention preferably contains a second "marker" gene that enables identification of the nucleic acid. This is most commonly used to distinguish the transformed plant cell containing the foreign nucleic acid from other plants cells that do not contain the foreign nucleic acid. Examples of such marker genes include antibiotic resistance, herbicide resistance and Glucuronidase (GUS) expression. Expression of the marker gene is preferably controlled by a second promoter which allows expression of the marker gene in cells other than those than dehiscence zones (if this is the tissue specific expression of the nucleic acid according to the first or second aspects of the

invention). Preferably the cell is from any of the Brassica family (most preferably *B. napus)*, maize or wheat or soyabean.

A fourth aspect of the invention provides a plant or a part thereof comprising a cell according to the third aspect of the invention. A whole plant can be regenerated from the single transformed plant cell by procedures well known in the art. The invention also provides for propagating material or a seed comprising a cell according to the third aspect of the invention. The invention also relates to any plant or part thereof including propagating material or a seed derived from any aspect of the invention.

A fifth aspect of the invention provides a signal transduction protein involved in the process of the plant dehiscence. The signal transduction protein according to the fifth aspect may have one or more of the preferred features according to the first or second aspects of the invention. Preferably it may be isolated, recombinant or in substantially pure form. It may comprise the various changes according to the first or second aspects. Preferably the protein is expressed from nucleic acid according to the first or second aspects. Alternatively, the protein can be provided using suitable techniques known in the art.

A sixth aspect of the invention provides a protein which;

a) comprises the amino acid sequence shown in figure 1 or;

b) has one or more amino acid deletions, insertions, or substitutions relative to a protein as defined in a) above and has at least 40% amino acid sequence identity therewith;

or a fragment of a protein as defined in a) or b) above which is at least 10 amino acids long.

The protein is preferably a signal transduction protein involved in the process of plant dehiscence and again, the preferred features of aspects one, two and five also applied to the sixth aspect.

The seventh aspect of the invention provides a process for regulating/controlling dehiscence in plant or in a part thereof, the process comprising obtaining a plant or a part thereof according to the fourth aspect of the invention. The process of dehiscence can be regulated and/or controlled by increasing or decreasing the expression of nucleic acid sequences according to the first or second aspect of the invention. Increased or decreased expression can easily be influenced by the person skilled in the art using technology well known. This includes increasing the numbers of copies of nucleic acid according to the invention in a plant or a plant thereof or increasing expression levels of copies of the nucleic acid present in particular parts or zones of the plant. Preferably the zones are dehiscence zones.

The process according to the seventh aspect of the invention includes obtaining a plant cell according to the third aspect of the invention or part of a plant according to the fourth aspect in the invention and deriving a plant therefrom. Alternatively, the process may comprise obtaining propagating material or a seed according to the fourth aspect of the invention and deriving a plant therefrom.

Preferably, the process of the seventh aspect of the invention is in the pod or the anther of a plant. All preferred features of aspects one to six also apply to the seventh.

An eighth aspect of the invention provides for the use of nucleic acid according to

the first to seventh aspects of the invention in the regulation/control of plant dehiscence. All preferred features of aspects one to seven also applies to the eighth.

The ninth aspect of the invention provides for the use of nucleic acid according to the first or second aspect of the invention as a probe. Such a probe can be used in techniques well known in the art to identify the presence of identical or homologous nucleic acid sequences from any source, preferably a plant source. The ninth aspect of the invention also provides nucleic acid identified by use of the nucleic acid from aspects one or two as a probe.

A tenth aspect of the invention provides for the use of nucleic acid according to aspects one or two of the invention in the production of a cell, tissue, plant or part thereof, or propagating material. Again, all preferred features of aspects one and two also apply to the tenth.

An eleventh aspect of the invention provides for nucleic acid comprising one or more of the underlined sequences as set out in figure 1. Such nucleic acid sequences are preferably used as primers in an PCR (Polymerase Chain Reaction) process in order to amplify nucleic acid sequences.

A twelfth aspect of the invention provides the use of nucleic acid according to the first or second aspects of the invention to identify another other protein or proteins which interact with its expression product. Such use can be carried out by the yeast two hybrid screening method (or others known in the art). The yeast two hybrid screening method is described for this aspect of the invention, in general, with reference to the sequence described as CW1. A potential way to implement the yeast 2-hybrid screen is outlined, as follows:

CW1 is linked to the Gal4 DNA binding domain and expressed in yeast which contains a pGAL4-lacZ gene. For activity of lacZ a second protein is required that contains the DNA transcriptional activation domain of GAL4 and that interacts with the CW1 protein. This is provided by making a cDNA expression library from plant DZ zones which results in fusions of plant proteins to the GAL4 activation domain. This library is transformed into the yeast strain that contains pGAL4-LacZ and expresses the CW1-Gal4 DNA bining domain protein fusion. Colonies that have lacZ activity are transformed with a gene for a protein that interacts with CW1.

Using such a system, upstream and downstream components of any signal transduction pathway can be identified, thus resulting in further ability to control/regulate dehiscence and/or male sterility.

A thirteenth aspect of the invention provides for a protein, as defined according to the limitations of the second aspect of the invention (without reference to figure 1) and nucleic acid encoding the protein, wherein the protein is capable of being identified according to the use (or method) according to the twelfth aspect of the invention.

A fourteenth aspect of the invention provides for the use of a protein according to the fifth or sixth aspect of the invention as a probe. In this context the probe is a means to identifying interacting entities (such as other proteins), including upstream and downstream interacting signal components. A protein according to the fifth or sixth aspect of the invention can be used as a probe to directly look for interactions with other proteins, i.e. purified protein can be used to look for complex formation with other plant protein, particularly isolated from the DZ zone. For example, a modified recombinant CW1 protein can be made with a sequence tag, such as a His-

tag, that enables the CW1 + interacting protein to be directly purified on a His affinity column. Alternatively, an antibody can be raised to CW1 protein. This antibody is then used to identify CW1 protein complexes and to purify the complexes. The CW1 interacting proteins can be purified and microsequenced to enable cloning of the genes for these interacting proteins.

The present invention provides a particularly useful method by which plant dehiscence can be regulated/controlled.

In addition to the use of the present invention in the production of shatter resistance or shatter-delayed plants such as oil seed rape, the invention may be used to control/regulate pollen release (by the control/regulation of anther dehiscence) which can produce male sterile plants. The temporal and spatial expression of nucleic acid encoding a protein according to the first and second aspects of the invention may require adjustment in obtaining the correct levels of dehiscence delay or prevention in different zones. For example, if pod dehiscence is required but anther dehiscence is not, it is necessary to ensure that expression of nucleic acid according to a first or second aspect of the invention has the correct temporal and spatial expression in order to obtain pod dehiscence or delay but not, to any substantial extent, anther dehiscence. This can be obtained by processes known in the art and may require use of particular promoter sequences to obtain the desired result. Usually in plant transformation, some difference in the level of expression of nucleic acid is observed in different plants. In some cases, the ratio of expression levels in different tissues can vary between different plant transformants thus providing essentially tissue-specific expression in one or other of the target tissues in some of the plant transformants. In the present invention, the natural expression of nucleic acid according to the first or second aspects may be predominantly higher in pod dehiscence zones and lower in the anther and funiculus dehiscence zones. However,

as described above, it is possible to obtain plants in which the protein expression is regulated in a particular dehiscence zone. Accordingly, a particularly useful aspect of the invention is the provision of plants which have one or both of the following features; are male sterile, are shatter resistant.

As described earlier, the process of dehiscence at the dehiscence zone involves the secretion of a number of enzymes, including hydrolytic enzymes. While previous attempts have been made to down or up regulate specific genes encoding particular proteins involved in the process of dehiscence, regulation by means of a signal transduction protein which effects expression of a number of genes is likely to be more effective than regulation of a single gene. In addition to this, the nucleic acid of the present invention has been identified as being expressed earlier than several other known genes involved in the process of plant dehiscence. This suggests that it is important earlier on in the process of plant dehiscence and can be used to control/regulate plant dehiscence at an earlier stage.

The nucleic acid encoding a signal transduction protein involved in the process of dehiscence or the signal transduction protein itself may be a component of a signal pathway that may either positively or negatively regulate pod shatter.

A more detailed explanation of such regulations/control, described with reference to a pod shatter (dehiscence) model is described below. As a skilled person will acknowledge, the model described below also relates to other general processes of dehiscence such as in the anther.

In the process of dehiscence, a particular signal transduction protein may be required to transmit a signal from the almost mature seed which initiates the expression or release of enzymes required for pod shatter. In this model, developmental signals

switch on expression and/or activation of a particular signal transduction protein in the pod dehiscence zone. This leads to expression of genes required for the release of pod dehiscence zone enzymes (such as hydrolytic enzymes). In this case, prevention of activity of the signal transduction protein, for example by downregulation of expression of this protein, would result in reduced dehiscence.

Alternatively, the developing seed may transmit a signal which represses the expression and/or activity of a particular signal transduction protein until late in cell development. In this model, developmental signals switch on a particular signal transduction protein which, in due course, represses the expression of genes required for release of dehiscence zone specific enzymes (such as hydrolytic enzymes). In this case, expression of a modified signal transduction protein that is constitutively active would result in reduced dehiscence.

A signal tranduction protein which is either positively or negatively involved in the process of dehiscence can be used according to the present invention.

The invention is described by reference to the enclosed drawings, in which;

Figure 1 CW1 full length sequence showing original PCR product and primer sites

Figure 2 Amino acid alignment with bacterial response regulator proteins & *ETR1*

Figure 3 Northern analysis of expression of CW1 in pods and other tissues. The lower panel shows the ethidium bromide-stained RNA gel prior to blotting and probing with CW1

Figure 4 Comparison of bacterial two-component systems with CW1

The present invention is now described with reference to the following, non-limiting examples.

Example 1- Isolation and characterisation of expression of CW1

Plant Material

Seeds of *B. napus* cv Rafal were grown as described by Meakin and Roberts, (1990a) with the following modifications. Single seedlings were potted into 10cm pots, and after vernalization, were re-potted into 21cm pots. At anthesis tags were applied daily to record flower opening. This procedure facilitated accurate age determination of each pod. Pods were harvested at various days after anthesis (DAA). The dehiscence zone was excised from the non-zone material and seed using a scalpel blade (Meakin and Roberts (1990b)) and immediately frozen in liquid N_2 and stored at -70°C.

RNA Isolation

All chemicals were molecular biology grade and bought from either Sigma Chemical Ltd (Dorset, UK), or Fisons (Loughborough, UK). Total RNA was extracted using the polysomal extraction method of Christoffersen and Laties, (1982), with the following alterations. The plant material was ground to a powder in liquid N_2 and then in 10 volumes of extraction buffer (200mM Tris-acetate [pH 8.2], 200mM magnesium acetate, 20mM potassium acetate, 20mM EDTA, 5% w/v sucrose, after sterilisation 2-mercaptoethanol was added to 15mM and cycloheximide added to a final concentration of 0.1 mg ml^{-1}). The supernatant was then layered over 8 ml 1M sucrose made with extraction buffer and centrifuged in a KONTRON™ (Switzerland) TFT 70.38 rotor at

45,000rpm (150,000g) for 2 hr at 2°C in a Kontron CENTRIKON™ T-1065 ultra-centrifuge. Pellets were then resuspended in 500µl 0.1M sodium acetate, 0.1% SDS, pH 6.0 and phenol/chloroform (1:1 v/v) extracted and the total RNA precipitated. Poly(A)$^+$ RNA was isolated from total RNA extracted, from both the zone and non-zone tissue of 40, 45 and 50 DAA pods, using a Poly(A) QUIK™ mRNA purification kit (Stratagene, Cambridge, UK) following the manufacturers instructions, and then bulked together. Total RNA was also extracted from leaves, stems, seeds and pods using a method described by Dean *et al*, (1985) for use in Northern analyses.

Differential display

This was performed essentially as described by Liang and Pardee (1992) using RNA extracted from 40 DAA pod dehiscence zones and non-zones. First strand cDNA copies of the RNAs (40 DAA DZ/NZ) were made using 50U M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (50U/µL) (Stratagene) in a 20µL reaction containing 1x M-MLV buffer, 2.5mM dNTPs (Pharmacia), 1µg RNA, 30U RNAse inhibitor (Promega) and 10µM oligo dT anchor primer 7 (5'-TTTTTTTTTTTTGG-3'). The reaction conditions were as follows: 65°C for 5 minutes, 37°C for 90 minutes and 95°C for 5 minutes. Following first strand cDNA synthesis, 60µL dH2O were added and the samples were either used directly for PCR or stored at -20°C.

For PCR, 2µL cDNA were used as template in a 20µL reaction containing 1x PCR buffer, 1mM $MgCl_2$, 2µM dNTPs, 10µM oligo dT anchor primer 7 (5'-TTTTTTTTTTTTGG-3'), 2.5µM arbitrary primer A (5'- AGC CAG CGA A –3'), 0.5µL 35S-dATP (> 1000 Ci/mmol) (Amersham) and 1U *Taq* DNA polymerase (5U/µL) (Gibco BRL). The thermocycling conditions were as follows: 40 cycles of 94°C for 30 seconds, 40°C for 2 minutes, 72°C for 30 seconds followed by 72°C for

5 minutes. The PCR products were fractionated on a 5% polyacrylamide/7M urea gel after addition of 5μL loading buffer (95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue) to each sample. Following electrophoresis the gel was dried at 80^{0}C under vacuum for 1 hour then exposed to X-ray film (BioMax-MR, Kodak) in a light tight cassette for 48 hours. The dried gel and autoradiogram were aligned so that bands that appeared in the DZ and not in NZ could be cut out and the DNA eluted according to Liang et al. (1995). The eluted PCR products (4μL) were reamplified in a 40μL reaction containing 1x PCR buffer, 1mM $MgCl_2$, 20μM dNTPs, 10μM oligo dT anchor primer 7 (5'-TTTTTTTTTTTTGG-3'), 2.5μM arbitrary primer A (5'- AGC CAG CGA A –3') and 2U *Taq* DNA polymerase (5U/μL) (Gibco BRL) using the following thermocycling conditions: 40 cycles of 94^{0}C for 30 seconds, 40^{0}C for 2 minutes, 72^{0}C for 30 seconds followed by 72^{0}C for 5 minutes. The resulting PCR product was cloned into the TA cloning vector (Invitrogen) and sequenced (Figure 1). In order to prepare an antisense strand-specific riboprobe, the PCR product was subcloned into pBluescript (Stratagene).

Expression analysis and characterisation of CW1

Northern analysis using an antisense strand-specific riboprobe to the CW1 PCR product, showed that CW1 hybridised to a transcript of 0.6kb which is expressed in the DZ of 20-50 DAA pods with a peak in expression at 40DAA. Minimal expression was observed in the pod NZ [Figure 2]. A random-primed labelled DNA probe (Stratagene) of the 330bp CW1 PCR product (amplified using primers CW1FL and CW1RL - see Figure 1) was used to screen a *B. napus* DZ cDNA library from which, following three rounds of screening to obtain pure plaques, a full length CW1 cDNA (606bp) was obtained (Figure 1).. An antisense strand-specific riboprobe of the full length CW1 cDNA was hybridised to total RNA

extracted from pod DZ/NZ (as in Figure 2), leaf abscission zones (AZ) and non-zones (NZ) (following exposure to 10μL/L ethylene for 72 hours), seed, root, flower and leaf. Figure 3 shows that CW1 hybridises to a 0.6kb message which is present in the pod DZ at 20-50 DAA with maximum expression at 40DAA. Again there is minimal expression in pod NZ and no apparent expression of CW1 in AZ, NZ, leaf, root, seed or flower RNA. By the sensitive technique of RT-PCR analysis CW1 expression can also be detected in anthers and the funiculus, both tissues that contain dehiscent zones

The 606bp cDNA (CW1) encodes a putative protein of 136 amino acids. Comparison of the CW1 translated sequence to the OWL protein database (Bleasby A.J., Attwood T.K., OWL – a nonredundant composite protein sequence database. Nucleic Acids Research, 22: 3574-3577 (1994)) showed low but consistent homology to a group of bacterial proteins comprising two-component regulatory systems. In particular, CW1 possesses the conserved amino acid residues required for phosphorylation of the receiver domain of the response regulator component (see Figure 4). CW1 plays a role in a signal transduction cascade resulting at least in one respect in pod shatter. It is therefore a good candidate for down-regulation of pod shatter processes using antisense technology. CW1 is a novel plant protein in that independant proteins with homology to bacterial receivers are yet to be reported in plants.

The full length cDNA was excised from the pBluescript cloning vector by digestion with *Eco*RI and *Xho*I restriction enzymes (Gibco BRL). Following purification from a 1% agarose gel the 606bp cDNA was random primed labelled (Stratagene) and used to screen a *B. napus* genomic library in the BlueStar vector. Following three rounds of screening to obtain pure plaques, a single genomic clone was isolated

which carries a 15kb genomic DNA insert. The promoter of the CW1 gene is isolated from this genomic clone using standard techniques.

Example 2 - Production of shatter-resistant *B.napus* plants by antisense downregulation of CW1

Downregulation of the CW1 gene or reduction in CW1 protein levels in the pod DZ will result in plants that are resistant (or more resistant than without this modification) to pod shatter. Standard techniques, commonplace in the art, such as the expression of antisense CW1 mRNA, full sense mRNA, partial sense mRNA or a ribozyme directed against CW1 mRNA are effective. Expression of these RNAs requires a promoter that is active in the pod DZ at the time at which CW1 is expressed. Ideally the promoter will be pod DZ-specific, however a useful promoter may be pod-specific or even constitutively active. A suitable promoter would be that of CW1. Although CW1 is expressed in the anther DZ, pod DZ and funiculus DZ, CW1 promoter -GUS fusion studies show that in different transformants the relative level of expression in these three sites is variable but is stability hereditable. Thus some transformants are obtained in which expression is largely or exclusively confined to the pod DZ. This suggests that the pCW1 promoter is comprised of distinct elements each specifying expression in a particular DZ. Alternatively the site of transgene integration may influence relative expression levels in the DZ tissues. The CW1 promoter is therefore linked to the CW1 cDNA such that the CW1 is in the antisense orientation forming pCW1-antiCW1. This chimeric gene is transferred to the binary vector pNos-NptII-SCV (W0 96/30529). This binary vector is transferred to the agrobacterial strain pGV2260 and transformed *B.napus* plants produced by agrobacterial transformation essentially as described in Moloney M et al., (1989) Plant Cell Reports 8, 238-242. A proportion of transformed *B.napus* plants exhibit reduced levels of CW1 message and are resistant to pod shatter.

REFERENCES

1. Christoffersen and Laties, Proc. Natl. Acad. Sci. 79, 4060-4063 (1982)

2. Dean *et al.*, EMBO J. 4: 3055-3061 (1985)

3. Hocking P J an Mason L: Accumulation, distribution and redistribution of dry matter and mineral nutrients in fruits of canola (oilseed rape) and the effect of nitrogen fertiliser and windrowing. Australian Journal or Agriculture Research 44: 1377-1388

4. Johnson-Flanagan AM and Spencer MS: Ethylene production during development of mustard *(Brassica juncea)* and canola (*Brassica napus)* seed. Plant Physiol 106: 601-606 (1994).

5. Liang P and Pardee A: Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257: 967-971 (1992).

6. Laing P, Bauer D, Averboukh L, Warthoe P, Rohrwild M, Muller H, Strauss M, Pardee A B: Analysis of altered gene expression by differential display. Methods in Enzymol 254: 304-321 (1995).

7. Meakin P J and Roberts J A: Dehiscence of fruit in oilseed rape *(Brassica napus* L.): anatomy of pod dehiscence. J Expt. Bot 41: 995-1002 (1990a).

8. Meakin P J and Roberts J A: Dehiscence of fruit in oilseed rape *(Brassica napus* L.): the role of cell wall degrading enzymes and ethylene. J Expt. Bot 41: 1003-1011 (1990b).

CLAIMS

1. Nucleic acid encoding a signal transduction protein involved in the process of dehiscence

2. Nucleic acid as claimed in claim 1 wherein the process involves the production of a hydrolytic enzyme

3. Nucleic acid as claimed in claim 1 or claim 2 which is naturally expressed in a dehiscence zone.

4. Nucleic acid encoding a protein wherein the protein:

   a) comprises the amino acid sequence shown in Figure 1 or;

   b) has one or more amino acid deletions, insertions or substitutions relative to a protein as defined in a) above and has at least 40% amino acid sequence identity therewith; or

   c) is a fragment of a protein as defined in a) or b) above, which is at least 10 amino acids long.

5. Nucleic acid as claimed in any one of claims 1 to 4 which comprises the sequence set out in Figure 1 or a fragment thereof which is at least 30 bases long.

6. Nucleic acid, as claimed in any one of claims 1 to 5 which is isolated or recombinant.

7. Nucleic acid which is antisense to nucleic acid as claimed in any one of claims 1 to 6.

8. Nucleic acid as claimed in any one of claims 1 to 7 including a promoter or other regulatory sequence which controls expression of the nucleic acid.

9. Nucleic acid which is the naturally occurring promoter or other regulatory sequence which controls expression of nucleic acid as claimed in any one of claims 1 to 8.

10. Nucleic acid as claimed in any one of claims 1 to 9 which is in the form of a vector.

11. A cell comprising nucleic acid as claimed in any one of claims 1 to 10.

12. A plant cell as claimed in claim 11.

13. A plant or a part thereof comprising a cell as claimed in claim 11 or claim 12.

14. Propagating material or a seed comprising a cell as claimed in claim 11 or claim 12.

15. A signal transduction protein involved in the process of plant dehiscence.

16. A protein which:

a) comprises the amino acid sequence shown in Figure 1 or;

b) has one or more amino acid deletions, insertions or substitutions relative to a protein as defined in a) above, and has at least 40% amino acid sequence identity therewith; or

c) a fragment of a protein as defined in a) or b) above which is at least 10 amino acids long.

17. A protein as claimed in claim 15 or claim 16 which is isolated or recombinant.

18. A process for regulating/controlling dehiscence in a plant or a part thereof, the process comprising obtaining a plant or part thereof as claimed in claim 13.

19. A process as claimed in claim 18 which comprises obtaining a plant cell as claimed in claim 12 or part of a plant as claimed in claim 13 and deriving a plant therefrom.

20. A process as claimed in claim 18 which comprises obtaining propagating material or a seed as claimed in claim 14 and deriving a plant therefrom

21. A process as claimed in claim 18 wherein the dehiscence is of a pod or of an anther.

22. Use of nucleic acid as claimed in any one of claims 1 to 10 in the regulation/control of plant dehiscence.

23. Use of nucleic acid as claimed in any one of claims 1 to 10 as a probe.

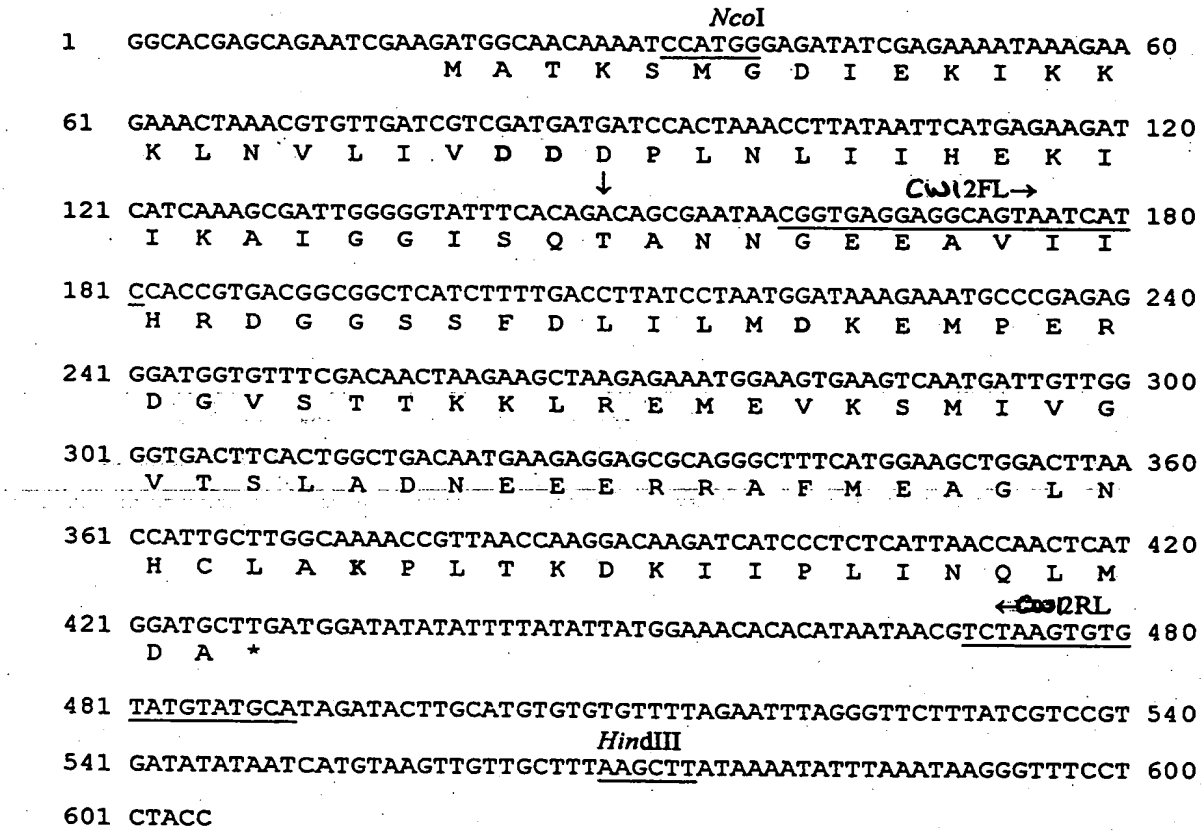
24. Use of nucleic acid as claimed in any one of claims 1 to 10 in the production of a cell, tissue, plant port thereof or propagating material.

25. Nucleic acid comprising one or more of the underlined sequences as set out in Figure 1.

26. Use of the nucleic acid as claimed in claim 25 as a PCR primer.

27. Use of a protein as claimed in any one of claims 15 to 17 as a probe.

Figure 1 - Nucleic acid and predicted protein sequence of CW2



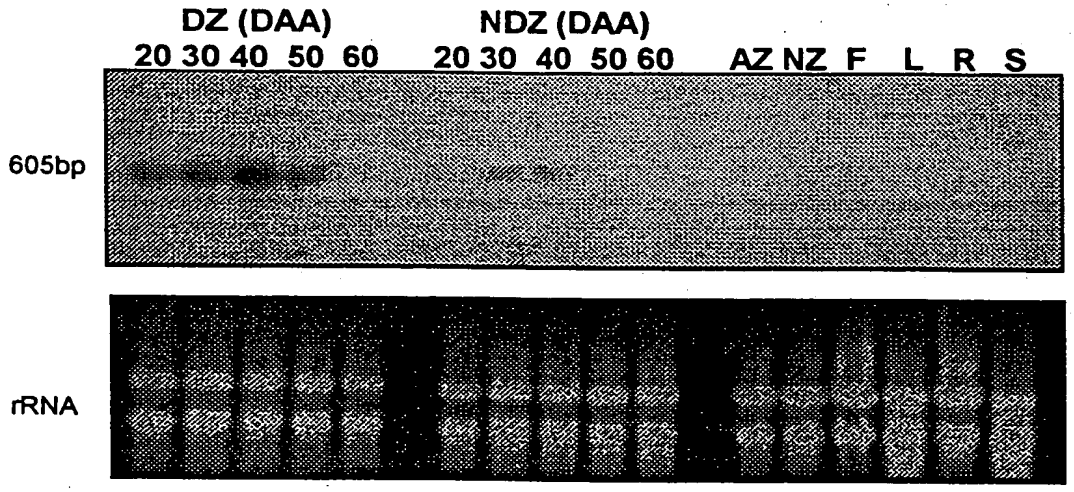
The primer sites for CW12FL and CW12RL are underlined, as are the recognition sequences for *Nco*I and *Hind*III restriction enzymes. Shown in bold are the conserved amino acid residues required for phosphorylation. The extent of the original PCR product isolated by differential display is shown by ↓.

Figure 2 - Alignment of the predicted protein sequence of CWI1 with those of bacterial response regulator proteins.

```
          1                                                  50
 CWI1     MATKSMGDIE KIKKKLNVLI VDDDPLNLII HEKIIKAIG. GISQTANNGE
 OMPR     .......... .MQENYKILV VDDDMRLRAL LERYLTEQGF .QVRSVANAE
 PHOB     .......... ...MARRILV VEDEAPIREM VCFVLEQNGF .QPVEAEDYD
 NTRC     .......... ..MQRGIVWV VDDDSSIRWV LERALAGAGL .TCTTFENGN
SPOOF     .......... ..MMNEKILI VDDQYGIRIL LNEVFNKEGY .QTFQAANGL
 CHEY     .......... MADKELKFLV VDDFSTMRRI VRNLLKELGF NNVEEAEDGV
  ETR     .......... .....LKVLV MDENGVSRMV TKGLLVHLGC EVTTVSSNEE

          51                                                 100
 CWI1     EAVIIHRDGG SSFDLILMDK EMPERDGVST TKKLREMEVK SM..IVGVTS
 OMPR     QMDRLLTR.. ESFHLMVLDL MLPGEDGLSI CRRLRSQS.. NPMPIIMVTA
 PHOB     SAVNQLNE.. PWPDLILLDW MLPGGSGIQF IKHLKRESMT RDIPVVMLTA
 NTRC     EVLAALAS.. KTPDVLLSDI RMPGMDGLAL LKQIKQ..RH PMLPVIIMTA
SPOOF     QALDIVTK.. ERPDLVLLDM KIPGMDGIEI LKRMKV..ID ENIRVIIMTA
 CHEY     DALNKLQA.. GGYGFVISDW NMPNMDGLEL LKTIRADGAM SALPVLMVTA
  ETR     ....CLRVVS HEHKVVFMDV CMPGVENYQI ALRI...... .HXPLLVALS

          101                                                150
 CWI1     LADNEEERRA FMEAGLNHCL AKPLTKDKII PLINQLMDA. ..........
 OMPR     KGEEVDRIVG .LEIGADDYI PKPFNPRELL ARIRAVLRRQ ANELPGAPS.
 PHOB     RGEEEDRVRG .LETGADDYI TKPFSPKELV ARIKAVMRRI SPM.......
 NTRC     HSDLDAAVSA .YQQGAFDYL PKPFDIDEAV ALVERAISHY QEQQQPRNIE
SPOOF     YGELDMIQES .KELGALTHF AKPFDIDEIR DAVKKYLPLK SN........
 CHEY     EAKKENIIAA .AQAGASGYV VKPFTPATLE EKLNKIFEKL GM........
  ETR     GNTDKSTKEK CMSFGLDGVL LKPVSLDNIR DVLSDLL... ..........

          151
 CWI1     ....
 OMPR     ....      OmpR  = E. coli (Involved in osmoregulation)
 PHOB     ....      PhoB  = E. coli (Involved in phosphate utilisation)
 NTRC     VNGP      NtrC  = S. typhimurium (Involved in nitrogen utilisation)
SPOOF     ....      SpoOF = B. subtilis (Involved in sporulation)
 CHEY     ....      CheY  = E. coli (Involved in chemotaxis)
  ETR     ....      ETR   = A. thaliana ETR1 gene encoding an ethylene receptor (partial amino acid
                            sequence)
```

The predicted protein sequence of CWI1 is shown in bold as are the conserved amino acid residues required for phosphorylation of the protein

Figure 3 - Expression analysis of CWI in various plant organs using Northerns



DZ = Pod dehiscence zone (20-60 DAA)
NDZ = Pod non-zone (20-60DAA)
AZ = Leaf abscission zone
NZ = Non-zone (stem)
F = Flower
L = Leaf
R = Root
S = Seed

Figure 4- Comparison of bacterial two-component regulatory systems with CW1